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ANTIBODY TECHNIQUES

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Contributors
Preface

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IAN M. ZITRON

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- II. Innate and Adaptive Immunity
- III. Acquired Immunity
A. The Formation of Clonal Populations
B. Immunological Memory
- IV. Primary and Secondary Responses
- V. The Temporal Course of the Immune Response

FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL SORTING

HYUN S. LILLEHOJ AND ALISON MARTIN

I. INTRODUCTION

Flow cytometry (FCM) is a method that measures the chemical and/or physical characteristics of particles as they pass through a detection device in a fluid stream. In immunofluorescence FCM, properties of the particles, usually cells, are assessed by an antibody molecule conjugated to a fluorochromatic dye. Fluorescence-activated cell sorting (FACS) is a process in which viable cells with pre-defined properties are separated by FCM. Biological particles that have been analyzed by FCM and FACS include prokaryotic and eukaryotic cells, normal and tumor cells, viruses, fungi, parasites, cellular organelles, and chromosomes. Commercially available flow cytometers include sophisticated optical, electronic, and nuclear radiation sensors as well as computers for data analysis.

With the advent of monoclonal antibodies against cell-surface antigens, FCM has evolved as a standard research tool. FCM analysis is useful not only to quantify distinct subpopulations of cells, but also to characterize the host's antibody response to bacteria, virus, or parasites. Most current immunological applications of flow cytometry relate to identification and quantification of heterogeneous cell populations. Also, analyses of physical characteristics (cell size and shape, cytoplasmic granularity, protein fluorescence, DNA content) as well as functional characteristics (enzyme activity, redox state, intracellular

ANTIBODY TECHNIQUES

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receptors, endocytosis, surface receptors, membrane-bound and cytoplasmic Ca^{2+} , intracellular pH) are possible using the flow cytometer.

One of the most popular applications of FCM is in the biological sciences, especially immunology, for isolation and characterization of subpopulations of cells within heterogeneous populations. Most successful experimental and clinical applications of flow cytometric analysis in medical practice are in the related fields of clinical immunology and hematology for a variety of tasks involving blood cell counting and classification. Applications are also being pursued in the fields of genetics, microbiology, oncology, parasitology, pharmacology, and toxicology.

Various types of commercial flow cytometers are available, but most of their basic functions are similar and can be described as follows. Single cells in liquid suspension are carried through a sensing device commonly illuminated by laser beams. In immunofluorescence FCM, fluorochrome molecules attached to antibody molecules bound to the biological particles are excited at a particular wavelength of light as the particles pass through a laser beam. The fluorochromes subsequently decay and emit light of another wavelength which is detected by the optical device. Light scattering, collected over one or more angular ranges, is determined and the light is converted into electronic signals by photomultiplier tubes (PMTs). Electronic signals whose magnitudes are proportional to the intensity of the emitted and scattered light are amplified and evaluated.

Fluorescein isothiocyanate (FITC) is generally used as a fluorochrome, and many antibodies conjugated to FITC are available commercially. Phycoerythrin (PE), another fluorochrome in general use, is excited at the same wavelength as FITC (Table I), thus providing a method of two-color FCM and FACS. PE labeling is more technically complex than FITC labeling and may not be practical for many laboratories. However, many

TABLE I Fluorochromes Used in FCM

Fluorochrome	Peak wavelength (nm)		Light source
	Absorption	Emission	
Fluorescein	495	530	Argon laser (488)
Rhodamine	555	620	Mercury arc (546)
B-Phycoerythrin	546	575	Mercury (546)
R-Phycoerythrin	490, 546	575	Argon (488)
Texas Red	568	590	Krypton laser (576)
Allophycocyanin	650	660	Helium-Neon laser (633)
Propidium iodide	490	585	Argon (488)

secondary antibodies labeled with fluorescein, Texas Red and allophycocyanin. The excitation wavelengths differ from those of the primary antibodies when autofluorescence is present or in cases of two-, three-

II. INSTRUMENTATION

An optical flow cytometer consists of a sensing device and illumination optics. Flow cytometers, however, are commonly used in light microscopy. The sensing device is typically illuminated by an intense light source (e.g., xenon lamps) or lasers.

A variety of instruments are available (Table II). For analytical cytology, the most common type of flow cytometer and cell sorter is the laser flow cytometer. The physical and chemical properties of the cell suspension, whereas a cell population. The types of laser used in the meter are the laser or the model of laser is usually different manufacturers offered are argon, krypton, and helium-neon lasers. In many flow cytometers, pressure mercury or xenon lamps are used. They are less expensive to purchase but are easier to incorporate in the instrument. The disadvantages, such as the low intensity of excitation wave, make it more difficult in shaping the instrument. For measuring light scattering, instruments also have different types. They are urged to contact various manufacturers to compare products before purchase.

Although commercial computer systems that collect data from these systems are available, they are obtained from the electrical signals of the scattered or fluorescent light in linear or logarithmic scale. The voltage is in 0.01-V increments (char-

membrane-bound and cytosolic components using the flow cytometer.

FCM is in the biological sciences and characterization of cell populations. Most successful flow cytometric analysis in clinical immunology and blood cell counting and used in the fields of genetics, pharmacology, and toxicology.

Many instruments are available, but most are described as follows. Single channel sensing device commonly used in FCM, fluorochrome excitation and emission spectra are similar to the biological particles. Light as the particles pass through the optical device. Light intensity is determined and recorded by photomultiplier tubes. Signal intensity is proportional to the concentration of the sample and is amplified and evaluated. FCM is used as a fluorochrome, and is available commercially. FCM, in general use, is excited at a wavelength providing a method of two-dimensional analysis. It is more complex than FITC analysis. However, many

secondary antibodies labeled with PE are commercially available. Texas Red and allophycocyanin require a second laser since their excitation wavelengths differ from those of FITC and PE. They are generally used when autofluorescence is a problem (e.g., with long-term cell cultures) or in cases of two-, three-, or four-color analyses.

II. INSTRUMENTATION

An optical flow cytometer includes a light source, light collection optics, and illumination optics similar to the objectives of a light microscope. Flow cytometers, however, require more intense light sources than commonly used in light microscopes since a cell passing through the instrument is typically illuminated for less than 10 μ sec. This requirement for an intense light source is met by arc lamps (high-pressure mercury and xenon lamps) or lasers.

A variety of instruments is commercially available worldwide (Table II). For analytical cytology, two major classes of instruments are available: flow cytometers and cell sorters. In general, a flow cytometer measures the physical and chemical characteristics of cells in a flowing liquid suspension, whereas a cell sorter can also selectively separate different cell populations. The two most important components of a flow cytometer are the laser or arc lamp and the flow chambers. The type and model of laser is usually specified by the manufacturer since lasers from different manufacturers have different sizes and shapes. Those currently offered are argon, krypton, xenon, and helium-neon ion lasers and dye lasers. In many flow cytometers, the excitation source consists of a high-pressure mercury or xenon arc lamp. Compared with lasers, arc lamps are less expensive to purchase and maintain, and are much smaller and easier to incorporate into instruments. However, they possess several disadvantages, such as lower light output at all wavelengths, less selectivity of excitation wavelength, relatively short life-span of the lamp, more difficulty in shaping and transporting the beam, and no possibility for measuring light scatter at small angles. The different commercial instruments also have different fluidics and optical arrangements. Buyers are urged to contact various manufacturers for detailed information and compare products before purchase.

Although commercial flow cytometers are equipped with various computer systems that collect, store, and analyze FCM data, the data received by these systems are essentially the same. The level of fluorescence obtained from the electronic sensors corresponds to the amount of scattered or fluorescent light and is translated into digitized readings either in linear or logarithmic fashion. These output voltages are recorded in 0.01-V increments (channels) from which histograms are constructed. It

Light source

Argon laser (488)
Mercury arc (546)
Mercury (546)
Argon (488)
Krypton laser (576)
Helium-Neon laser (633)
Argon (488)

TABLE II Commercial Flow Cytometers

Source	Comments
<p>Becton Dickinson Immunocytometry Systems 2375 Garcia Avenue P.O. Box 7375 Mountain View, CA 94039 800-821-9796</p>	<p>Becton Dickinson Immunocytometry Systems include the FACS Analyzer and FACScan and the latest versions of the Fluorescence Activated Cell Sorter (FACS®) which are called the FACStar and the FACStar Plus. The Becton Dickinson cell sorters use a water-cooled argon ion laser source (2-5 Watts), and a Cyomics air-cooled argon ion laser.</p>
<p>Coulter Corporation Epics Division P.O. Box 4486 Hialeah, FL 33014-0486 305-885-0131</p>	<p>EPICS® instruments are laser source flow cytometers with an orthogonal geometry. The EPICS V is designed primarily for research laboratory use, whereas EPICS C and EPICS Profile are intended for clinical applications. The standard laser in the EPICS research instruments is a water-cooled 4-W argon laser with enhanced UV output. A second ion laser and/or dye laser can be added for dual-beam illumination. The EPICS C is a single beam sorting instrument using a 2-W argon laser. The EPICS Profile is a nonsorting bench-top flow cytometer system designed for routine clinical laboratory applications and the standard light source is an air-cooled Omnicrome argon laser emitting about 25 mW at 488 nm.</p>
<p>Kratel SA 64 Ch. de St. Maurice/BP82 CH-1222 Geneva-Vesenez Switzerland (022) 52 33 74</p>	<p>The Kratel Partograph flow cytometers include the Partograph FMP Analyzer and Partograph FMP Sorter. The Partograph FMP is available with low power (15 mW) argon and/or He-Ne laser sources. An arc lamp source and sorting capability are also offered.</p>
<p>ODAM 34 Rue de L'Industrie 67160 Wissenbourg France 94 99 32</p>	<p>The ODA ATC 3000 flow cytometer is a multiparameter cell sorter equipped with argon and krypton ion lasers or dye lasers in a dual system.</p>
<p>Skatron A/S P.O. Box 8 N-3401 Liet Norway (03)850770</p>	<p>The Argus flow cytometer is the recent commercial version of the arc source instrument. Argus Flow Microphotometer is equipped with a 100-W mercury arc lamp. An argon laser is optional.</p>

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The power of flow cyton
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III. APPLICATIONS

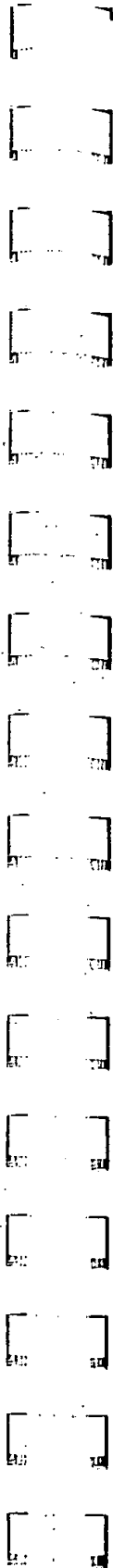
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ODAM
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94 99 32

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(03)850770

The ODAM ATC 3000 flow cytometer is a multiparameter cell sorter equipped with argon and krypton ion lasers or dye lasers in a dual system.

The Argus flow cytometer is the recent commercial version of the arc source instrument. Argus Flow Microphotometer is equipped with a 100-W mercury arc lamp. An argon laser is optional.



is desirable in some systems, especially when more than two measurements are made on each cell, to collect data in list mode so that the full set of measurements can be repeatedly processed for optimal results.

Data are normally collected for forward scatter (cell size), side scatter (cell internal complexity), and one or more fluorescence wavelengths. Data analysis can use many forms of display and numerical evaluation, including single parameter histograms in which cell counts are plotted as a function of the signals collected by a single sensor and contour plots and dot displays showing cell frequency distribution as a function of measurements by two FACS sensors. Sample statistics, such as the percentage of cells falling within specified ranges of sensor readings and the means of signal levels, can also be acquired.

One-parameter FCM data can be displayed simply by plotting the number of cells with a particular signal strength as a function of the signal strength. The y axis shows the number of cells falling into each channel whereas the x axis is usually divided into 256 channels. The resulting bar graph is usually drawn with only the tops of the bars connected to form a broken line, where the height under any part of the line reflects the total count of cells in a particular channel. It is thus possible to visually estimate the relative frequencies of each cell type in a mixed population.

Typical two-parameter FCM displays contain information about the frequency of cells for two measurement parameters. Channel values for each parameter are presented on the x and y axes. In a dot display, the number of dots in each region simply indicates the cell count. Contour plots are similar to a geological survey topographic map in which contour lines are used to define specified cell frequency levels.

Both linear and logarithmic signal amplifications are available for most flow cytometers. The choice of which to use has important implications for the display and analysis of FACS data. Although both logarithmic and linear data displays contain the same information, linear amplification is appropriate for signals with a limited dynamic range and for signal distributions that include narrow peak components, such as DNA data. In contrast, logarithmically processed data can cover a wide dynamic range at constant relative resolution and are suitable for some biological measurements such as the distribution of a cell-surface antigen among cell populations.

III. APPLICATIONS

The power of flow cytometric analysis lies in its ability to make quantitative multiparameter measurements on a large number of individual cells. Any of a number of cellular properties can be detected with a fluorescent

label or antibody; that measurement, in combination with other cytological properties, provides a sophisticated characterization of the cell. Particularly with the advent of monoclonal antibody technology, FCM has provided a method of rapid quantitative analysis of multiple cellular antigens.

Examples of FCM applications of importance in cell biology include quantitative cell-cycle analysis, flow karyotyping and chromosome sorting, flow cytometry and sorting of sperm cells, virus-cell interaction analysis, and measurements of intracellular ionized calcium concentration and membrane potential. In basic and clinical immunology, FCM has been important for correlation of changes in particular cell subpopulations with the onset of clinical disease, investigation of T and B lymphocyte developmental pathways, identification of functional characteristics of lymphocyte subpopulations, and, most recently, studying the regulation of gene expression. In the field of hematology, FCM has contributed to analysis and sorting of normal and malignant blood and bone marrow cells.

IV. PROCEDURES

For further reference, the reader should consult Shapiro (1988); Parks *et al.* (1989); Melamed *et al.* (1990); Coligan *et al.* (1991); and Robinson (1993).

A. Preparation of Reagents

Lysis Reagent

NH ₄ Cl	8.2 g
NaHCO ₃	0.84 g
EDTA	0.37 g
Sterile distilled water	1 liter

Adjust pH to 7.2; store at 4°C for 1 mo.

2% Paraformaldehyde

Paraformaldehyde	10 g
Balanced salt solution	500 ml

Warm to 60°C to improve solubility. Adjust pH to 7.2 with NaOH before adjusting final volume to 500 ml. Store in the dark at 4°C for 1 mo.

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10% Sodium Azide

Sodium azide
Balanced salt solution

Store at room temperature

FCM Buffer

10% Sodium azide
Heat-inactivated fetal calf serum
Balanced salt solution

Store at 4°C for 1 mo
substituted for fetal calf serum

Sheath Fluid

Normal saline (0.85% NaCl)
filtered.

B. Preparation of Single Cells

1. Preparation of Cells from Tissue

- Following the protocol for tissue dissociation, wash the tissue with ethanol to sterilize lymph nodes.
- Place organ(s) in a sterile container with Hank's balanced salt solution.
- Disrupt the tissue by syringe plunger or aspirator.
- Transfer to a 100- μ m mesh screen to filter the resulting single cell suspension. Adjust the volume to 10 ml with sheath fluid.
- Centrifuge at 2000 rpm for 5 min.
- Wash the pellet twice by centrifuging as in Step 5.
- Resuspend cells in culture medium.

2. Preparation of Tumor Cells

- Cell dissociation for flow cytometry depends on the nature of the tumor.

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ance in cell biology include ing and chromosome sorting, virus-cell interaction, ionized calcium concentration, immunology, FCM in particular cell subpopulation of T and B lymphocytes, studying the regulation, FCM has contributed to blood and bone marrow

alt Shapiro (1988); Parks *et al.* (1991); and Robinson

ust pH to 7.2 with NaOH. Store in the dark at 4°C

10% Sodium Azide

Sodium azide	10 g
Balanced salt solution	100 ml

Store at room temperature for 2 mo.

FCM Buffer

10% Sodium azide	10 ml
Heat-inactivated fetal calf serum	30 ml
Balanced salt solution	1 liter

Store at 4°C for 1 mo. Bovine serum albumin (1%, w/v) may be substituted for fetal calf serum.

Sheath Fluid

Normal saline (0.85%) made in double-distilled water and triple filtered.

B. Preparation of Single Cell Suspensions

1. Preparation of Cells from Lymphoid Organs of Animals

1. Following the protocol established for humane sacrifice of the animal, wet with ethanol to sterilize and remove spleen, thymus, and/or lymph nodes.
2. Place organ(s) in a sterile petri plate containing a small volume of Hank's balanced salt solution (HBSS) with 5% fetal calf serum.
3. Disrupt the tissue by mincing with scissors, then mashing with a syringe plunger or aspirating up and down with a syringe and needle.
4. Transfer to a 100- μ m screen and filter to remove clumps. Transfer the resulting single cell suspension to a centrifuge tube and bring volume to 10 ml with HBSS.
5. Centrifuge at 2000 rpm for 5 min.
6. Wash the pellet twice by resuspending the cells in 10 ml HBSS and centrifuging as in Step 5.
7. Resuspend cells in culture medium and count by trypan blue exclusion.

2. Preparation of Tumor Cells

1. Cell dissociation for flow cytometry should be achieved enzymatically for preservation of cell morphology and function. Enzymes used will depend on the nature of the tumor, and some experimentation may

be needed. Suggested enzymes include trypsin, collagenase, neutral protease, DNase, and hyaluronidase, or combinations thereof. In general, sarcomas are more easily digested than carcinomas.

2. Follow the procedure given for single cell suspensions. After the tissue is minced with scissors, incubate with appropriate enzyme(s) for 1 hr. Filter out clumps and continue as in Procedure 1.

3. Preparation of Leukocytes by Lysis of Erythrocytes

1. Obtain blood sample, or prepare a single cell suspension of lymphoid organ cells.
2. Pellet cells by centrifugation and resuspend in 5 ml lysing reagent per organ, per 2 ml blood, or per $5-10 \times 10^7$ viable lymphocytes.
3. Incubate 10 min at room temperature with gentle mixing.
4. Fill tube with HBSS and centrifuge at 2000 rpm for 5 min. Wash the pellet with HBSS twice and resuspend cells in culture medium.

4. Ficoll-Hypaque Isolation of Mononuclear Cells

1. If using whole blood, dilute in an equal volume of HBSS, or prepare a single cell suspension of organ cells in HBSS.
2. Place 4 ml Ficoll-Hypaque in 17×100 -mm tubes.
3. Overlay carefully with cell suspension, 5-6 ml per tube.
4. Centrifuge at 1800 rpm at room temperature for 20 min.
5. Transfer lymphocytes from the interface to an appropriately sized tube and dilute at least 50% in HBSS.
6. Centrifuge at 2000 rpm for 5 min. Wash cells twice with HBSS by resuspending pellet in 10 ml or more of HBSS and centrifuging at 2000 rpm for 5 min.

C. Basic Cell Staining Procedure

1. Antibodies

1. For detailed protocols to conjugate antibody to FITC or biotin, see Chapter 9. To conjugate antibody to Texas Red, follow the FITC labeling procedure using 1 mg/ml Texas Red in cold anhydrous acetonitrile.

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2. Use fluorochrome-conjugated antibody. For some applications, use a spacer between the fluorochrome and the antigen-binding site to prevent precipitation.
3. For biotin-conjugated antibodies, use streptavidin-conjugated bovine serum albumin.

2. Cell Staining

1. Keep all cells, antibodies, and reagents at 4°C. Avoid capping of antibodies.
2. Prepare a single cell suspension in cell culture medium.
3. Determine viable cell count.
4. Centrifuge cell suspension at 2000 rpm for 5 min. Resuspend in 1 ml supernatant. Resuspend on ice.
5. Distribute 50 μ l cell suspension into each well of a microtiter plate.
6. Add 50 μ l antibody to each well. If the antibody is unlabeled, use a predetermined amount. A few unstained control cells are added to each well to determine cytometer settings.
7. Mix antibody with cells for 30 min, depending on the antibody.
8. Centrifuge plates at 2000 rpm for 5 min. Flick once to discard supernatant.
9. Wash the cells with 1 ml HBSS. Gently mix the buffer as in Step 8.
10. If appropriate, add 50 μ l of secondary antibody to each well (with specificity for the primary antibody). Incubate for 30 min.
11. Resuspend in 500 μ l HBSS. Distribute into microtiter plates, resuspend.

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2. Use fluorochrome-conjugated avidin to detect biotin-labeled anti-
body. For some applications, it may be necessary to use biotin con-
taining a spacer between the protein-binding and avidin-binding sites
to prevent precipitation.
3. For biotin-conjugated antibodies, use FCM buffer prepared with 1%
bovine serum albumin instead of fetal calf serum.

2. Cell Staining

1. Keep all cells, antibodies, and buffers on ice at all times to prevent
capping of antibodies.
2. Prepare a single cell suspension of cells as described earlier and place
in cell culture medium on ice.
3. Determine viable cell count by trypan blue exclusion.
4. Centrifuge cell suspension for 8 min at 1800 rpm and 4°C, and discard
supernatant. Resuspend in FCM buffer at 2×10^7 cells/ml and keep
on ice.
5. Distribute 50 μ l cell suspension to small tubes (13-mm) or wells of
a microtiter plate.
6. Add 50 μ l antibody to each tube or well. For a direct assay, this
antibody will be FITC labeled; for an indirect assay, the primary
antibody is unlabeled. Appropriate dilutions of antibody should be
predetermined by testing intensity of staining with several dilutions.
A few unstained controls should be prepared for optimizing the flow
cytometer settings.
7. Mix antibody with cells by shaking gently. Incubate on ice 20-
30 min, depending on the affinity of the antibody.
8. Centrifuge plates or tubes 5 min at 1800 rpm and 4°C. Decant or
flick once to discard supernatant.
9. Wash the cells with 100-200 μ l ice-cold FCM buffer 2-3 times by
gently mixing the buffer with cells, followed by 5 min centrifugation
as in Step 8.
10. If appropriate, add 50 μ l FITC-labeled avidin or second antibody
(with specificity for the primary antibody). Include half of the control
samples from Step 6 to measure nonspecific binding of second anti-
body to cells. Incubate 20-30 min on ice. Wash as in Step 9.
11. Resuspend in 500 μ l ice-cold FCM buffer. If staining was done in
microtiter plates, resuspend in 100 μ l FCM buffer and then add to

tubes containing 400 μ l ice-cold FCM buffer. Keep on ice until flow cytometry.

12. If cells will be stored before running, fix by resuspending in 100 μ l FCM buffer and, while vortexing, adding 100 μ l 2% paraformaldehyde solution. Keep samples at 4°C for up to several days.

D. Two-Color Cell Staining

The steps for two-color staining are the same as those for single color staining, with the following exigencies.

1. When direct staining, both antibodies may be added simultaneously if there will be no interactions between them.
2. When using one direct and one indirect antibody, add the indirect primary antibody first. If both antibodies are of the same species, it may be necessary to perform a blocking step before adding the direct antibody. To do this, after the excess secondary antibody has been washed off, an excess of purified immunoglobulin of the same species as the primary antibody is added and incubated for 10 min prior to adding the antibody specific for the surface antigen. The excess immunoglobulin occupies any unused binding sites of the secondary antibody.
3. When both antibodies will be indirectly labeled, the assay must be arranged to avoid any possibility of interactions between them.
 - a. If the primary antibodies are of different species, they may both be added during the first incubation. Then the secondary antibodies specific for each species may be added during the second incubation, as long as they will not bind each other or cross-react with other species.
 - b. If any of the antibodies are of the same species, it will be necessary to conduct sequential incubations. Adjust the order in which antibodies are added to avoid interactions and use blocking steps (Step 2) as necessary.
4. Prepare control samples that are singly stained with each fluorochrome to use when adjusting the flow cytometer. If a dual-positive control is available, it should be stained with both fluorochromes.

E. Operation of Flow Cytometer

The instructions here are generalized and must be used in conjunction with the manufacturer's instructions.

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1. Turn on machine 20 min before use.
2. Check levels of sheath fluid as necessary. Add a disinfectant for disinfection.
3. Flush air from the tubing.
4. Run the wash cycle using distilled water or sheath fluid. Be included in these washes more than 200 events per run.
5. When laser is warmed up, perform alignment. Some alignment is required prior to running. Follow the manufacturer's directions. On the alignment beads should show fluorescence peaks showing the signal while minimizing the batch of fluorescence. Coordinates may be saved for subsequent runs.
6. Run a sample of unstained cells/sec or within machine.
7. Adjust the forward scatter. The choice between linear and logarithmic all parameters may be made. Linear cell population range is limited. More logarithmic cell populations compress the range of the signal.
8. Adjust side scatter PMT voltage. Necessary to achieve good separation, for example, to distinguish lymphocytes.
9. Adjust PMT voltages. Stained cells, adjust PMT voltage that the peak of unstained histogram or on the fluorescence amplification gains, then adjust the logarithmic gains of FL1 and FL2 and compress the scale.

buffer. Keep on ice until flow

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1. Turn on machine 20 min to 1 hr before use to warm up the laser.
2. Check levels of sheath fluid and waste in reservoirs; empty and fill as necessary. Add a small amount of bleach to the waste reservoir for disinfection.
3. Flush air from the tubing.
4. Run the wash cycle using 5% bleach to degrade proteins, followed by distilled water or sheath fluid. Manufacturer's cleansers may also be included in these washes. Following wash, there should be fewer than 200 events per min.
5. When laser is warmed up, run fluorescent beads and check alignment. Some alignment beads specify that they should not be vortexed prior to running. Adjust alignment according to manufacturer's directions. On the forward scatter vs side scatter histogram, the alignment beads should fall in a tight cluster. The variability of the fluorescence peaks should be small (1–2%). The aim is to maximize the signal while minimizing variability. Once the ideal parameters for the batch of fluorescent beads have been established, these coordinates may be saved and used to adjust the alignment of the beads for subsequent runs.
6. Run a sample of unstained cells and adjust flow rate to 1000–2000 cells/sec or within manufacturer's recommended parameters.
7. Adjust the forward scatter amplification gains to exclude cell debris. The choice between linear and logarithmic amplification gains for all parameters may be made on the basis of the homogeneity of the cell population. Linear amplifications are appropriate for homogeneous cell populations or for cell cycle analyses in which the intensity range is limited. More information may be obtained on heterogeneous cell populations by using logarithmic amplification gains to compress the range of intensity.
8. Adjust side scatter PMT voltages and amplification gains as necessary to achieve good separation of different cell types within a sample, for example, to distinguish granulocytes from monocytes and lymphocytes.
9. Adjust PMT voltages and amplification gains. While running unstained cells, adjust PMT voltage of fluorescence 1 (FL1) and FL2 so that the peak of unstained cells falls either off the left side of the histogram or on the far left side, as preferred. If using logarithmic amplification gains, these parameters may not be adjustable. Logarithmic gains of FL1 and FL2 will increase the scale of weak signals and compress the scale of strong signals.

10. While running stained cells, adjust PMT voltages of FL1 and FL2 to achieve good separation from the peak of unstained cells.
11. Run stained cell sample and adjust regions, bitmaps, or gates. Bitmaps can be drawn around each type of cell in a nonhomogeneous cell population, such as whole blood. Alternatively, if only one cell population is used, bitmaps may be drawn in concentric rings to compare those cells that are most alike as well as the larger population containing more variability. Likewise, regions of interest in single-parameter histograms may be adjusted to include various proportions of the peak from all the cells to only those that stain most intensely.
12. Run the test samples.
13. Run the wash cycle as in Step 5.
14. Initiate the shutdown process for the machine.
15. Gates, bitmaps, and regions are used to identify cells of interest. In general, gates refer to one-dimensional thresholds whereas bitmaps are two-dimensional. These parameters are applied to data acquisition; regions are thresholds applied to data analysis. Gates on data acquisition mean that only cells of interest will be included in the cell count. For example, it is desirable to set a gate on forward scatter or side scatter (or both) to exclude cell debris. Gates and bitmaps can also be set to include only the cells of interest from a mixed cell population. It is often helpful to set a cut-off for fluorescence obtained by examining unstained cells, or to determine the fluorescence produced by nonspecific binding of an irrelevant antibody or the secondary antibody of an indirect procedure.

F. Propidium Iodide Elimination of Dead Cells

1. Make 25–50 $\mu\text{g}/\text{ml}$ propidium iodide (PI) in PBS.
2. Stain cells and optimize machine for single staining protocol, as described.
3. Run unstained sample, and set up FL1 (FITC) vs FL2 (PI) histogram.
4. Adjust PMT of FL2 so cells appear in lower left quadrant (perhaps 10–20 V less than FL1).
5. Run FITC-stained sample. Adjust compensation by increasing the setting for %FL1 in FL2. This increases the %FL1 that is subtracted from the FL2 sensor, until the histogram shows no higher readings for FL2 in stained than unstained cells.

6. Add 10 μl PI solution to control sample.
7. Set a gate for FL2 to exclude the dead PI-stained cells.
8. Add 10- μl PI to FITC-stained sample to verify the validity of the FL2 gate.
9. Add 10 μl PI to each sample.
10. After running all samples, add bleach, followed by centrifugation and washing.

G. Two-Color FCM Analysis

1. Stain cells with FITC and PE. In addition, make a control sample, using cells and reagents, stain a dual-positive sample.
2. Warm up machine and run unstained cell parameter.
3. Run unstained sample.
4. Adjust FL2 so cells appear in lower left quadrant.
5. Run FITC-stained control sample. Set %FL1 in FL2 setting to 10%.
6. Run the PE-stained control sample. Set %FL1 in FL2 setting to 10%.
7. Run the dual-positive sample. Confirm that the %FL1 in FL2 setting is 10% vs FL2 histogram.
8. Mix the single-stained samples to insure that no dual-positive cells are present.
9. Run samples.

V. PITFALLS AND COMPLICATIONS

Optimal labeling of antibodies is critical. All reagents should be used. Texas Red labeling is most common.

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6. Add 10 μ l PI solution to unstained control. Vortex and run the control sample.
7. Set a gate for FL2 to include the unstained control cells but exclude the dead PI-stained cells.
8. Add 10- μ l PI to FITC-stained sample. Vortex and run. Confirm the validity of the FL2 gate for the positive cells.
9. Add 10 μ l PI to each sample immediately prior to running it.
10. After running all samples, be sure to run the wash cycle using 5% bleach, followed by distilled water, to clean excessive dye from the tubing.

G. Two-Color FCM Analysis

1. Stain cells with FITC and PE, following the two-color staining protocol. In addition, make single-stained control samples for both FITC and PE, using cells and antibodies that stain most brightly. If possible, stain a dual-positive control sample with FITC and PE.
2. Warm up machine and adjust for fluorescent beads, controls and unstained cell parameters, and PMT voltages for FL1 and FL2.
3. Run unstained sample, and set up FL1 vs FL2 histogram.
4. Adjust FL2 so cells appear in lower left quadrant.
5. Run FITC-stained control. Adjust compensation by increasing the %FL1 in FL2 setting until the FL2 reading is no more than for unstained cells.
6. Run the PE-stained control. Adjust compensation of %FL2 in FL1 until the FL1 is no more than for unstained cells.
7. Run the dual-positive control sample stained with both FITC and PE. Confirm that the cells fall in the upper right quadrant of the FL1 vs FL2 histogram.
8. Mix the single-stained control samples from Steps 5 and 6 and run to insure that no dual-positives are detected.
9. Run samples.

V. PITFALLS AND CONCERNS

Optimal labeling of antibodies with fluorochromes depends on the reagents used. All reagents of FITC and Texas Red should be made fresh. Texas Red labeling is more difficult than labeling with FITC or biotin,

since it is more dependent on the isotypes and species of antibody to be labeled. The ability of specific antibodies to withstand the conjugation procedures and retain activity should also be considered.

Obtaining high specific signal with minimal background fluorescence requires reducing background fluorescence caused by nonspecific binding and autofluorescence. Autofluorescence often occurs with cultured cells and can be reduced by using cells in an exponential growth phase, appropriate optical filters, or reagents that have been conjugated with red fluorochromes. Unstained cells should be included as controls to assess background autofluorescence. A high fluorochrome-to-protein ratio in the conjugation procedure can improve signal-to-noise ratios. Fixation of cells in dilute solutions of paraformaldehyde may increase the nonspecific background fluorescence.

All experiments should include negative controls labeled with irrelevant antibodies to determine nonspecific binding of any labeled reagent to the cell population under examination. For indirect staining, either omit the first antibody or use an irrelevant antibody of the same isotype to establish background fluorescence or nonspecific binding.

The intensity and specificity of staining can be influenced by several factors. It is important to include sodium azide in the staining buffer, and all the reagents and staining tubes should be kept on ice to decrease metabolic activity and inhibit the capping phenomenon. The optimum predetermined antibody concentration should be used in all staining procedures. All antibody preparations should be free of aggregates to avoid nonspecific binding to the Fc receptor present on the surfaces of some cells. Most secondary antibodies are polyclonal antisera that have been affinity purified. Optimum concentrations for each secondary reagent must be determined for each primary antibody used in each assay.

The choice of direct or indirect staining procedures requires a tradeoff. Direct staining of cell-surface markers with specific antibodies conjugated to FITC provides a more specific but less intense staining reaction. Indirect procedures, involving primary antibodies against cell-surface markers and secondary antibodies conjugated to FITC and directed against the primary antibody (usually in a species-specific manner), provide more intensity but with a loss of specificity. Signal intensity may also be enhanced by conjugating the antibody to biotin and using FITC-conjugated avidin.

Cross-reactions are most problematic in multicolor staining systems in which two primary antibodies produced in different species are detected with species-specific secondary antibodies labeled with different fluorochromes. Controls in which each second-step reagent is tested on both primary antibodies individually must be included. The order of

adding reagents is critical for both direct and indirect staining. Every multicolor staining procedure in which cells are stained separately, then compared with single-color reagents, requires single-color control experiments for cytometric analysis.

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types and species of antibody to be able to withstand the conjugation process should be considered.

imal background fluorescence caused by nonspecific binding often occurs with cultured cells in an exponential growth phase, it has been conjugated with fluorescein isothiocyanate (FITC) and should be included as controls to determine the effect of fixation on the fluorescence-to-protein ratio. Fixation with formaldehyde may increase the

controls labeled with irrelevant reagent. For indirect staining, either antibody of the same isotype as specific binding.

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adding reagents is critical in multicolor staining. In a protocol in which both direct and indirect staining are performed, the indirect is done first. Every multicolor staining procedure should include single color controls in which cells are stained with each fluorochrome-labeled reagent separately, then compared with a sample stained with multicolor-labeled reagents. Single-color controls are used for setting compensation in flow cytometric analysis.

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